Review Article

Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol 2016;26:165–179
DOI: 10.1159/000440881

Published online: March 10, 2016

Fermentative Cyclohexane Carboxylate Formation in *Syntrophus aciditrophicus*

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Key Words
Cyclohexane carboxylate · Fermentation · *Syntrophus aciditrophicus*

Abstract
Short-chain fatty acids such as acetic, propionic, butyric or lactic acids are typical primary fermentation products in the anaerobic feeding chain. Fifteen years ago, a novel fermentation type was discovered in the obligately anaerobic Deltaproteobacterium *Syntrophus aciditrophicus*. During fermentative growth with crotonate and/or benzoate, acetate is formed in the oxidative branch and cyclohexane carboxylate in the reductive branch. In both cases cyclohexa-1,5-diene-1-carboxyl-CoA (Ch1,5CoA) is a central intermediate that is either formed by a class II benzoyl-CoA reductase (fermentation of benzoate) or by reverse reactions of the benzoyl-CoA degradation pathway (fermentation of crotonate). Here, we summarize the current knowledge of the enzymology involved in fermentations yielding cyclohexane carboxylate as an excreted product. The characteristic enzymes involved are two acyl-CoA dehydrogenases specifically acting on Ch1,5CoA and cyclohex-1-ene-1-carboxyl-CoA. Both enzymes are also employed during the syntrophic growth of *S. aciditrophicus* with cyclohexane carboxylate as the carbon source in coculture with a methanogen. An investigation of anabolic pathways in *S. aciditrophicus* revealed a rather unusual pathway for glutamate synthesis involving a *Re*-citrate synthase. Future work has to address the unresolved question concerning which components are involved in reoxidation of the NADH formed in the oxidative branch of the unique cyclohexane carboxylate fermentation pathway in *S. aciditrophicus*.

Introduction

The degradation of organic compounds in anoxic environments is of crucial importance for the global carbon cycle. In habitats with no or only trace amounts of electron acceptors, microbial metabolic pathways exist that substitute for respiratory processes. The degradation of complex natural polymers such as polysaccharides, proteins, nucleic acids and lipids often results in the formation of reduced low-molecular fermentation products such as short-chain alcohols or aliphatic/aromatic fatty acids like butyrate, propionate, lactate or benzoate. They can serve as substrates for secondary fermentations in bacteria, yielding acetate, H_2 and formate that serve as

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Table 1. Standard Gibbs free energy changes of *S. aciditrophicus* growing in coculture with a methanogen with different carbon sources

Benzoate	
$4\ C_7H_5O_2^- + 19\ H_2O \rightarrow 12\ C_2H_3O_2^- + 3\ CH_4 + HCO_3^- + 9\ H^+$	$\Delta G^{\circ\prime} = -201.5$ kJ total reaction $\Delta G^{\circ\prime} = -50.4$ kJ/mol substrate
Crotonate	
$4\ C_4H_5O_2^- + HCO_3^- + 5\ H_2O \rightarrow 8\ C_2H_3O_2^- + CH_4 + 3\ H^+$	$\Delta G^{\circ\prime} = -243$ kJ total reaction $\Delta G^{\circ\prime} = -60.8$ kJ/mol substrate
Cyclohexane carboxylate	
$2\ C_7H_{11}O_2^- + HCO_3^- + 5\ H_2O \rightarrow 6\ C_2H_3O_2^- + 3\ CH_4 + 3\ H^+$	$\Delta G^{\circ\prime} = -153$ kJ total reaction $\Delta G^{\circ\prime} = -76.4$ kJ/mol substrate
Cyclohex-1-ene-1-carboxylate	
$4\ C_7H_9O_2^- + HCO_3^- + 13\ H_2O \rightarrow 12\ C_2H_3O_2^- + 5\ CH_4 + 7\ H^+$	$\Delta G^{\circ\prime} = -401$ kJ total reaction $\Delta G^{\circ\prime} = -100.3$ kJ/mol substrate

The free energy changes were calculated with the values taken from Thauer et al. [1977], except for the values for cyclohexane carboxylate (–281.35 kJ/mol) and cyclohex-1-ene-1-carboxylate (–223.58 kJ/mol), which were calculated using the group contribution method [Mavrovouniotis, 1990].

substrates for methanogenic archaea [McInerney et al., 2009]. These processes are thermodynamically unfavorable under standard conditions but may become favorable by the immediate consumption of H_2, formate or acetate by methanogenic archaea. The necessary interaction between acetate-, H_2- and formate-producing and consuming species is referred to as syntrophy.

The Gram-negative, rod-shaped Deltaproteobacterium *Syntrophus aciditrophicus* SB has been established as a model organism for the investigation of anaerobic degradation of aromatic compounds under syntrophic growth conditions. It was isolated from a wastewater treatment plant with benzoate as the sole carbon source in coculture with the hydrogen-consuming sulfate-reducing *Desulfovibrio* G-11 [Hopkins et al., 1995], but it can also be cultivated syntrophically with *Methanospirillum hungatei* JF-1 [Elshahed et al., 2001]. Initially, *S. aciditrophicus* was reported to grow syntrophically with different aliphatic saturated fatty acids [Jackson et al., 1999], which, however, could not be easily reproduced (own unpubl. results). The four confirmed carbon sources used by *S. aciditrophicus* are crotonate and the cyclic fatty acids cyclohexane carboxylate, cyclohex-1-ene-1-carboxylate (cyclohexene carboxylate) and benzoate. While syntrophic growth in association with the formate- or hydrogen-consuming *M. hungatei* was reported for all four substrates, only crotonate and benzoate were shown to serve as the sole carbon source for axenic growth [Elshahed and McInerney, 2001; Mouttaki et al., 2007, 2008].

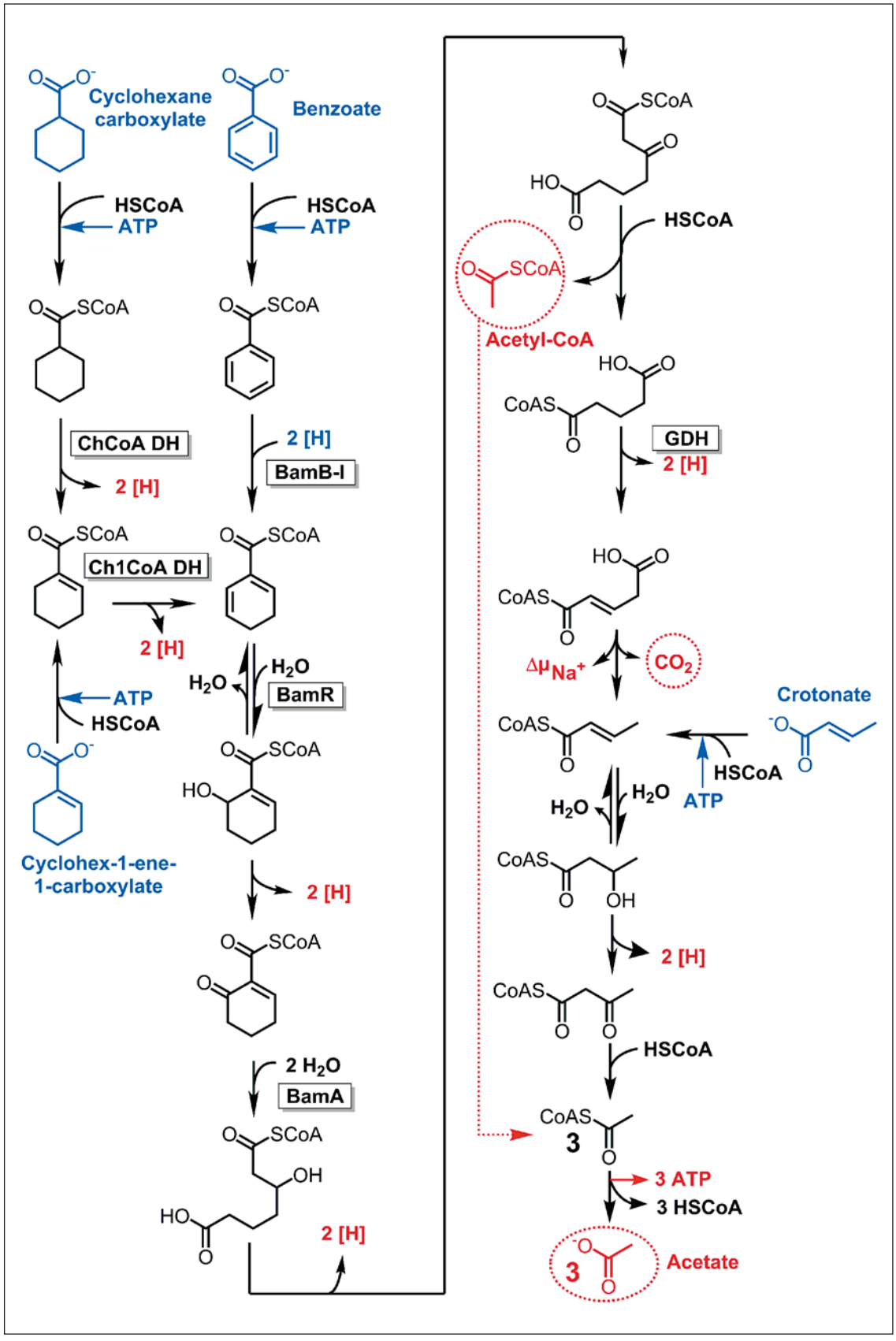
Syntrophic Growth of *S. aciditrophicus*

The stoichiometry of substrates consumed/products formed and the overall free energy changes during the growth of *S. aciditrophicus* with the four carbon sources under syntrophic conditions yielding acetate and methane are shown in table 1. Notably, the free energy change during benzoate degradation to cyclohexane carboxylate, methane and CO_2 yields only 10–15% of the energy compared to that with nonaromatic growth substrates.

The availability of the genome sequence of *S. aciditrophicus* in 2007 [McInerney et al., 2007], in conjunction with numerous biochemical studies, allowed insights into the enzymology involved in the syntrophic degradation of all four carboxylic acids. A benzoate CoA ligase activity was measured in extracts of cells grown syntrophically with benzoate [Elshahed et al., 2001]. In agreement, the genome contains several copies of genes encoding putative ATP-dependent CoA ligases, and it is

Fig. 1. Central catabolic pathways of *S. aciditrophicus* growing syntrophically with different carbon sources. Incoming compounds, reducing equivalents and ATP-consuming steps are depicted in blue, with outgoing compounds and ATP-forming reactions in red. Experimentally confirmed activities and genes of enzymes identified are shown in boxes. The reducing equivalents formed are consumed by the syntrophic partner, e.g. by reducing CO_2 to methane.

(For figure see next page.)


Cyclohexane carboxylate
Benzoate
HSCoA
ATP
HSCoA
ATP
SCoA
SCoA
ChCoA DH
2 [H]
2 [H]
BamB-I
SCoA
SCoA
Ch1CoA DH
2 [H]
H2O
H2O
BamR
ATP
HSCoA
SCoA
Cyclohex-1-ene-1-carboxylate
2 [H]
SCoA
2 H2O
BamA
SCoA
OH
2 [H]
SCoA
HSCoA
Acetyl-CoA
HO
CoAS
GDH
2 [H]
HO
CoAS
ΔμNa+
CO2
Crotonate
CoAS
HSCoA
ATP
H2O
H2O
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OH
2 [H]
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CoAS
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3 ATP
3 HSCoA
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Acetate

1

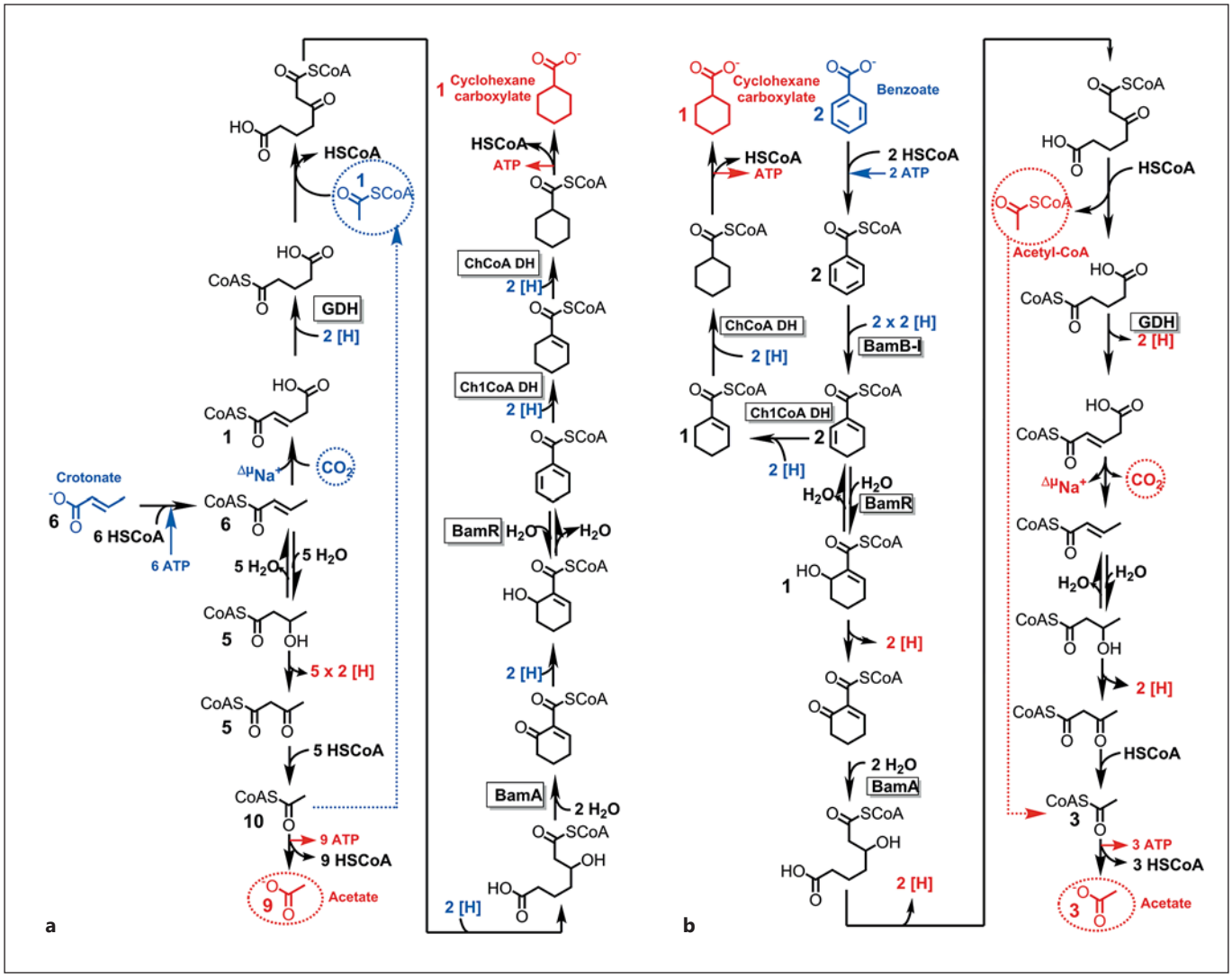

Fig. 2. Metabolic pathway of cyclohexane carboxylate formation during axenic growth of *S. aciditrophicus* with crotonate (**a**) and benzoate (**b**) as the sole carbon source. The numbers at individual compounds refer to stoichiometry for balanced electron transfer. Ingoing compounds, reducing equivalents and ATP-consuming steps are depicted in blue, with outgoing compounds and ATP forming steps in red. Experimentally confirmed activities and genes of enzymes identified are shown in boxes.

likely that crotonate, cyclohexane carboxylate and cyclohexene carboxylate are all activated by the corresponding gene products. Several genes encoding characteristic enzymes of the benzoyl-CoA degradation pathway originally identified in the facultatively anaerobic *Thauera aromatica* [Breese et al., 1998] or in the obligately anaerobic *Geobacter metallireducens* [Peters et al., 2007; Wischgoll et al., 2005] have also been found in *S. aciditrophicus* [McInerney et al., 2007] (fig. 1). For reductive benzoyl-CoA dearomatization *S. aciditrophicus* uses a class II benzoyl-CoA reductase (BCR) as evidenced by the presence of several copies of the encoding *bamBCEDFGHI* genes [McInerney et al., 2007]; genes encoding ATP-dependent class I BCRs are absent in the genome. In agreement, the electron acceptor-dependent aromatization of the product cyclohexa-1,5-diene-1-carboxyl-CoA (Ch1,5CoA) to benzoyl-CoA, was determined in vitro; such a reaction is typical for reversible class II but not for irreversible class I BCRs [Löffler et al., 2011]. The presence of further characteristic enzymes such as Ch1,5CoA hydratase (BamR), 6-oxocyclohex-1-ene-1-carboxyl-CoA hydrolase (BamA) and glutaryl-

Table 2. Standard Gibbs free energy changes of *S. aciditrophicus* growing axenically with different carbon sources

Crotonate	
$6\ C_4H_5O_2^- + HCO_3^- + 5\ H_2O \rightarrow 9\ C_2H_3O_2^- + C_7H_{11}O_2^- + 3\ H^+$	$\Delta G^{\circ\prime} = -289$ kJ total reaction $\Delta G^{\circ\prime} = -48.1$ kJ/mol substrate
Benzoate	
$2\ C_7H_5O_2^- + 7\ H_2O \rightarrow 3\ C_2H_3O_2^- + C_7H_{11}O_2^- + HCO_3^- + 3\ H^+$	$\Delta G^{\circ\prime} = -24.8$ kJ total reaction $\Delta G^{\circ\prime} = -12.4$ kJ/mol substrate
Crotonate and benzoate	
$3\ C_4H_5O_2^- + C_7H_5O_2^- + 6\ H_2O \rightarrow 6\ C_2H_3O_2^- + C_7H_{11}O_2^- + 3\ H^+$	$\Delta G^{\circ\prime} = -157$ kJ total reaction $\Delta G^{\circ\prime} = -52.3$ kJ/mol substrate
Cyclohex-1-ene-1-carboxylate	
$6\ C_7H_9O_2^- + 7\ H_2O \rightarrow 3\ C_2H_3O_2^- + 5\ C_7H_{11}O_2^- + HCO_3^- + 3\ H^+$	$\Delta G^{\circ\prime} = -220$ kJ total reaction $\Delta G^{\circ\prime} = -36.6$ kJ/mol substrate

The free energy changes were calculated with the values taken from Thauer et al. [1977], except for the values for cyclohexane carboxylate (–281.35 kJ/mol) and cyclohex-1-ene-1-carboxylate (–223.58 kJ/mol), which were calculated using the group contribution method [Mavrovouniotis, 1990].

CoA dehydrogenase by enzyme activity assays were determined in cell extracts and/or with heterologously produced proteins [Djurdjevic, 2010; Kuntze et al., 2008; Peters et al., 2007]. The enzymes responsible for the oxidation of crotonyl-CoA to acetyl-CoA involve standard crotonase, 3-hydroxyacyl-CoA dehydrogenase and thiolase, which should be active during growth with all four substrates [Elshahed et al., 2001].

Axenic Growth of *S. aciditrophicus*

Crotonate as Carbon Source

While initial studies suggested butyrate as a fermentation product during the axenic growth of *S. aciditrophicus* with crotonate [Jackson et al., 1999], recent publications identified cyclohexane carboxylate as the only reduced end product [Mouttaki et al., 2007]. Its formation can be explained by a stoichiometric oxidation of crotonate (C4) to acetate coupled to its concomitant reduction to cyclohexane carboxylate (C7); the reductive pathway involves two C-C bond-forming steps catalyzed by a carboxylase and a thiolase (fig. 2a). This fermentative pathway serves as an alternative route for a syntrophic interaction and guarantees growth/survival even in the absence of a syntrophic partner. Indeed, calculations of the overall free energy changes reveal that the fermentative formation of cyclohexane carboxylate and acetate from crotonate is clearly exergonic. Though not experimentally verified, axenic fermentation of cyclohexene carboxylate to acetate and cyclohexane carboxylate should thermodynamically also be feasible (table 2).

The degradation pathway of crotonate to acetate (oxidative branch) and cyclohexane carboxylate (reductive branch) was investigated by mass spectrometric analyses of metabolites [Mouttaki et al., 2007] and by in vitro activity measurements of characteristic enzymes (e.g. BamA, BamB and BamR) [Djurdjevic, 2010; Kuntze et al., 2008; Löffler et al., 2011; Peters et al., 2007]. In summary, the data obtained indicate that for axenic crotonate degradation, the reductive branch employs the typical enzyme inventory of the benzoyl-CoA degradation pathway leading from crotonyl-CoA to Ch1,5CoA in the reverse direction. However, the enzymes involved in the conversion of Ch1,5CoA to cyclohexane carboxylate are not part of this pathway and have only recently been identified and studied in some detail. Moreover, it remained unclear whether the exergonic reactions catalyzed by the ring cleaving BamA and the membrane-bound, sodium-dependent glutaconyl-CoA decarboxylase operate in the reverse reaction at sufficient rates. Results from recent studies addressing these issues are presented below.

Benzoate as Carbon Source

The axenic growth of *S. aciditrophicus* with benzoate as the sole carbon source has experimentally been demonstrated [Elshahed and McInerney, 2001]. Substrate

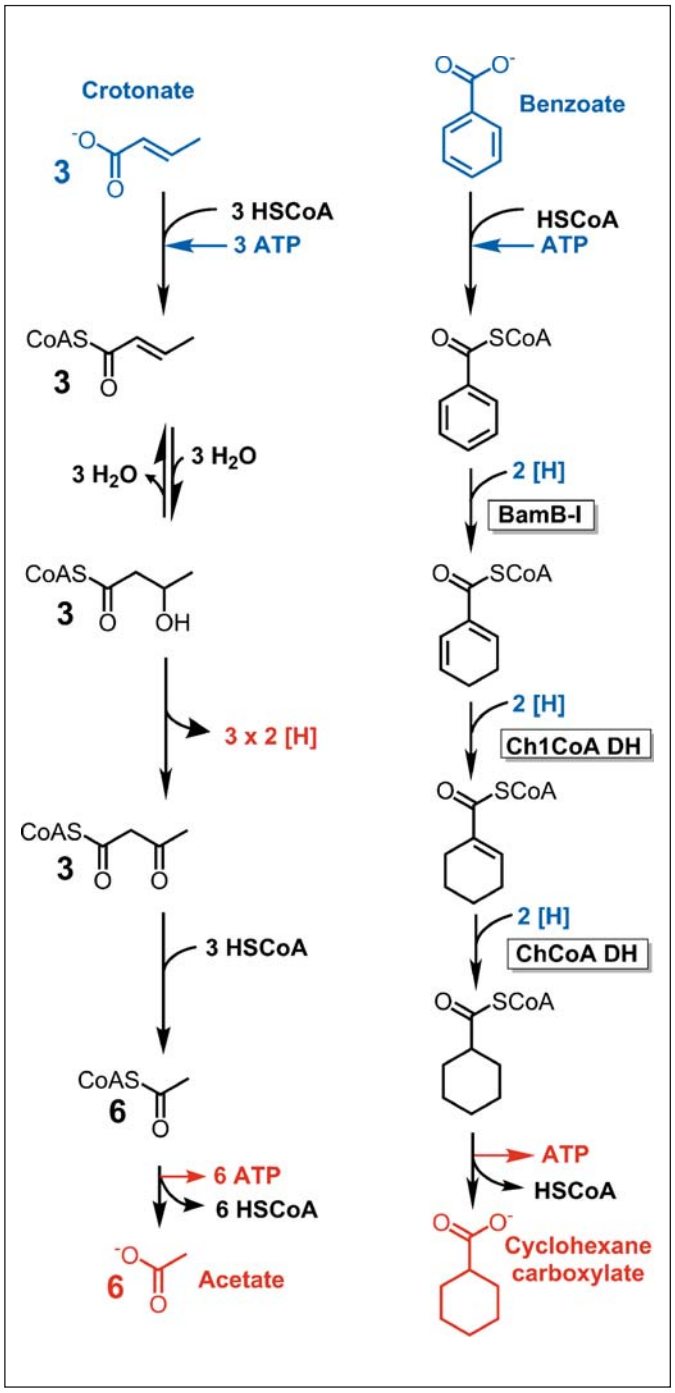

Fig. 3. Catabolic pathway of cyclohexane carboxylate formation during axenic growth of *S. aciditrophicus* with crotonate and benzoate as the mixed carbon source. The numbers at individual compounds refer to stoichiometry for balanced electron transfer. Ingoing compounds, reducing equivalents and ATP-consuming steps are depicted in blue, with outgoing compounds and ATP-forming reactions in red. Experimentally confirmed activities and genes of enzymes identified are shown in boxes. Reducing equivalents generated during crotonate oxidation to acetate are reoxidized during benzoate reduction to cyclohexane carboxylate.

consumption/product formation analyses suggested a disproportionation of two benzoate to three acetate and CO_2 (oxidative branch), and to one cyclohexane carboxylate (reductive branch; fig. 2b). However, calculations for the Gibbs free energy values vary due to the inaccuracy of the Gibbs free energy values of cyclohexane carboxylate for its formation from the elements (ΔG_f°; table 2). In any case, axenic growth with benzoate as the sole carbon and energy source is extremely difficult to observe but may be stimulated by the addition of small amounts of crotonate.

Mixed Fermentation of Benzoate and Crotonate

During growth with a mixture of ^{13}C-labeled benzoate and varying amounts of nonlabeled crotonate, all cyclohexane carboxylate formed is derived from labeled benzoate [Mouttaki et al., 2008]. Thus, reducing equivalents formed during the oxidation of crotonyl-CoA to two acetyl-CoA were recycled by the reduction of benzoyl-CoA to cyclohexane carboxyl-CoA (ChCoA) without using the enzymes involved in the reaction sequence from crotonyl-CoA to Ch1,5CoA that are employed during axenic growth with crotonate (fig. 3) [Mouttaki et al., 2008]. This finding is remarkable, because BCR is expected to depend on a reduced ferredoxin ($E' \approx -500$ mV) as electron donor [Kung et al., 2010], and the redox potential of the NADH formed during crotonyl-CoA oxidation is not sufficiently negative to serve as a donor for benzoyl-CoA reduction. A ferredoxin:NAD^+ oxidoreductase (ion-translocating Rnf complex), of which the encoding genes are present in the *S. aciditrophicus* genome, may catalyze a reduction of ferredoxin by NADH, albeit at the expense of the membrane potential [Biegel and Müller, 2010].

Characteristic Enzymes Involved in Cyclohexane Carboxylate Fermentation

During axenic growth with crotonate and/or benzoate, cyclohexane carboxylate is formed from Ch1,5CoA, a common cyclic intermediate of the respective pathways (fig. 2, 3). The enzymatic steps that convert this intermediate to cyclohexane carboxylate have only recently been studied, and a 1,2-adding as well as an unusual 1,4-adding acyl-CoA dehydrogenase were characterized [Kung et al., 2013, 2014] (fig. 4). In the following, the current knowledge and hypotheses of the enzymes involved in the reaction sequence from Ch1,5CoA to cyclohexane carboxylate are summarized.

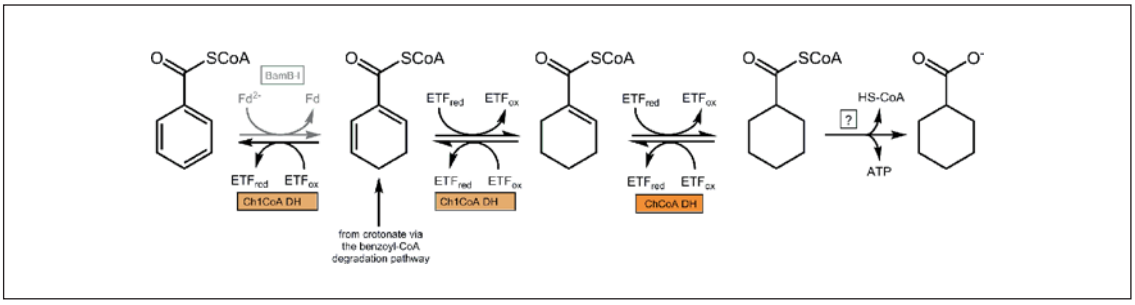

Fig. 4. Reactions catalyzed by ChCoA and Ch1CoA dehydrogenase. DH = Dehydrogenase. The activities were determined in the reverse direction using the artificial acceptor ferricenium hexafluorophosphate ($FcPF_6$); in the forward reaction they can, except for the first step, be measured with Ti(III)-citrate as an artificial electron donor. During axenic growth the acyl-CoA deyhdrogenases are employed in the forward direction for reoxidation of the reducing equivalents. The electrons are most likely transferred to/from the acyl-CoA dehydrogenases via ETF. Ch1CoA dehydrogenase can reduce Ch1,5CoA to benzoyl-CoA in a most likely unphysiological reaction. The reduction of benzoyl-CoA during growth with benzoate is catalyzed by a class II BCR [Fuchs et al., 2011]. The enzyme involved in the formation of cyclohexane carboxylate from ChCoA is unknown.

ChCoA Dehydrogenase

ChCoA was suggested as the final CoA-ester intermediate in the reductive branch of the cyclohexane carboxylate fermentation pathways, which is formed by the reduction of cyclohex-1-ene-1-carboxyl-CoA (Ch1CoA). A ChCoA dehydrogenase was purified via several chromatographic steps from extracts of *S. aciditrophicus* cells grown axenically with crotonate, and its encoding gene was identified as *SYN_02586* [Kung et al., 2013]. The homotetrameric enzyme has a native molecular mass of around 155 kDa and is composed of 42-kDa subunits. Typically for acyl-CoA dehydrogenases, it contains one FAD per subunit. The enzyme activity was determined by following the ferricenium hexafluorophosphate ($FcPF_6$)-dependent oxidation of ChCoA to Ch1CoA. The highly specific enzyme did not use butyryl-CoA, glutaryl-CoA or Ch1CoA as substrates. For measuring the reduction of Ch1CoA to ChCoA that is relevant growth with crotonate and/or benzoate, Ti(III)-citrate served as an artificial electron donor [Kung et al., 2013].

Ch1CoA Dehydrogenase

The nonreactivity of Ch1CoA dehydrogenase with Ch1,5CoA suggests that an additional enzyme must exist in cyclohexane carboxylate-forming cells that specifically converts Ch1,5CoA to Ch1CoA. A candidate gene, *SYN_02587*, annotated as a gene encoding acyl-CoA dehydrogenase with 44% amino acid sequence identities to ChCoA dehydrogenase from *S. aciditrophicus*, is located adjacent to the ChCoA dehydrogenase-encoding gene. Heterologous expression and purification by affinity chromatography yielded a yellow, tetrameric 150-kDa product with a stoichiometric FAD content (one per 38-kDa subunit) [Kung et al., 2013]. The enzyme specifically catalyzed the $FcPF_6$-dependent oxidation of Ch1CoA to Ch1,5CoA, which was, after prolonged incubation, further oxidized to benzoyl-CoA. No other common acyl-CoA substrates such as ChCoA or butyryl-CoA were accepted. Ch1,5CoA dehydrogenase catalyzed the Ti(III)-citrate-dependent reduction of Ch1,5CoA to Ch1CoA, which represents the physiologically relevant direction during growth with crotonate and/or benzoate, albeit by the use of an artificial reductant. The minor traces of benzoate that were usually detected in growth media of *S. aciditrophicus* during growth in pure culture with crotonate may result from the rather nonphysiological aromatization of Ch1,5CoA to benzoyl-CoA, catalyzed by Ch1,5CoA dehydrogenase in the presence of an appropriate acceptor [Elshahed and McInerney, 2001; Kung et al., 2010; Mouttaki et al., 2007]. Notably, the Ch1,5CoA intermediate is expected to be in equilibrium with the hydrated 6-hydroxycyclohex-1-ene-1-carboxyl-CoA, catalyzed by the specific Ch1,5CoA hydratase (BamR), a specific enzyme of the benzoyl-CoA degradation pathway [Peters et al., 2007].

The reaction catalyzed by Ch1CoA dehydrogenase is unusual for an acyl-CoA dehydrogenase as it represents a 1,4-addition to the conjugated diene system of Ch1,5CoA rather than the common 1,2-additions of standard acyl-CoA dehydrogenases (fig. 4). The latter contain a highly conserved, catalytic glutamate in either the central or C-terminal domain of 1,2-adding acyl-CoA dehydrogenases [Thorpe and Kim, 1995]. A sequence alignment of Ch1CoA dehydrogenase from *S. aciditrophicus* with

1,2-adding acyl-CoA dehydrogenases revealed that these glutamates are replaced by an asparagine and threonine, which cannot act as a catalytic base/acid [Kung et al., 2015].

To shed light on the apparently noncanonical catalytic mechanism, the crystal structure of Ch1CoA dehydrogenase in complex with Ch1CoA was solved from the closely related *G. metallireducens* [Kung et al., 2015]. In *G. metallireducens* the enzyme plays a role in the complete degradation of cyclohexane carboxylate to CO_2 coupled to respiratory Fe(III) or nitrate reduction [Kung et al., 2014]. Accordingly, the ring moiety of the reduced Ch1CoA was bound into the expected active site position sandwiched between the isoalloxazine ring of FAD and Thr363, Asn241, Ile87 and Asp91 (online suppl. fig. S1; see www.karger.com/doi/10.1159/000440881 for all online suppl. material). Thr363 and Asn241 occupy the expected glutamate positions in 1,2-dehydrogenating acyl-CoA dehydrogenases. Asp91 is the only residue in the active site and is perfectly positioned to serve as a proton acceptor/donor from/to the C3 of the substrate ring. A D91N mutant exhibited a complete loss of activity, which confirms the function of Asn91 as a proton acceptor/donor I in the Ch1,5CoA dehydrogenase reaction. Moreover, the substrate is oriented in a manner that its closest distance to FAD of 3.2 Å is between its C6 and the isoalloxazine N5.

On this basis, a catalytic mechanism for the oxidation of Ch1CoA to Ch1,5CoA is postulated, which is initiated by a deprotonation of the substrate C3 by Asp91 carboxylate, resulting in a delocalized anionic transitions state of the thioester substrate. Its conversion to the Ch1,5CoA is then accomplished by a hydride transfer from the substrate C6 to the N5 of the FAD isoalloxazine ring system. Taking into account the high amino acid sequence similarities and the invariance of catalytic amino acids, an identical 1,4-dehydrogenation mechanism is expected for the Ch1CoA dehydrogenase from *G. metallireducens* and *S. aciditrophicus* [Kung et al., 2015].

Conversion of ChCoA to Cyclohexane Carboxylate

In the last step of cyclohexane carboxylate fermentation the free carboxylic acid is released from the energy-rich thioester. Due to the poor energy yield of *S. aciditrophicus*, an energy-conserving reaction was anticipated. Three possible enzymes could be involved in such a process: (1) a CoA transferase, e.g. transferring the CoA moiety to the carboxylic acid growth substrate, (2) an ADP phosphorylating kinase in conjunction with a phosphotransacetylase and (3) an AMP- or ADP-dependent CoA ligase operating in the reverse reaction that synthesize ATP either from ADP + P_i or AMP + PP_i. Experimental evidence has been provided for neither of these enzymes. Instead, a ChCoA hydrolyzing thioesterase was enriched via several chromatographic steps from cells grown with crotonate [Kung and Boll, unpubl. data]. The 110-kDa protein contained 2.2 Fe per subunit and is annotated as 'rubrerythrin and thioesterase superfamily protein' *(SYN_02559)*, but due to the rather low activities in cell extracts (26 nmol/min/mg) it is unclear whether this enzyme is indeed responsible for hydrolyzing ChCoA. Moreover, the energy of the thioester substrate would be wasted by such a reaction, which is hardly conceivable for an organism thriving at the thermodynamic limit of life.

Electron Transfer from NADH to Acyl-CoA Dehydrogenases

During axenic growth with crotonate and/or benzoate the NADH formed in the oxidative branches has to transfer electrons to the electron-accepting ChCoA and Ch1CoA dehydrogenases. In the case of axenic growth with crotonate, a third acyl-CoA dehydrogenase, glutaryl-CoA dehydrogenase, is involved in NADH reoxidation (fig. 2a). In all three cases the reduction of the enoyl-CoA substrates ($E^{\circ\prime} \approx -10$ mV) with NADH ($E^{\circ\prime} = -320$ mV) is largely exergonic and could energetically be conserved by an electron-transferring protein (ETF)-mediated flavin-based electron bifurcation process [Buckel and Thauer, 2013; Herrmann et al., 2008]. Such a process was originally described for the ETFs from *Clostridium kluyveri* [Li et al., 2008] and *Acidaminococcus fermentans* [Chowdhury et al., 2014] that both form complexes with butyryl-CoA dehydrogenases. They couple the endergonic reduction of crotonyl-CoA by NADH with the endergonic reduction of ferredoxin by the same donor. An Rnf complex could then apply the exergonic reduction of NAD^+ by reduced ferredoxin to proton transport across the cytoplasmic membrane (fig. 5). It has to be considered, however, that under syntrophic conditions with benzoate, cyclohexane carboxylate or cyclohex-1-ene carboxylate as substrates, as well as under axenic conditions with benzoate or cyclohex-1-ene carboxylate as substrates, glutaryl-CoA dehydrogenase has to work in the oxidative direction (tables 1, 2). NAD^+ has to oxidize glutaryl-CoA to glutaconyl-CoA driven by the oxidation of reduced ferredoxin. Under standard conditions with $E^{\circ\prime} = -10$ mV for the glutaconyl-CoA/glutaryl-CoA couple and $E^{\circ\prime} = -420$ mV for ferredoxin ox/red, $\Delta G^{\circ\prime}$ becomes too high, which makes the oxidation of glutaryl-CoA impossible:

$2NAD^+ + glutaryl\text{-}CoA + ferredoxin_{red}^{2-} \rightarrow 2NADH + glutaconyl\text{-}CoA + ferredoxin_{ox}$

$\Delta E^{\circ\prime} = (2 \times -320\ mV) - [(-10\ mV) + (-420\ mV)] = -210\ mV$; $\Delta G^{\circ\prime} = +41\ kJ/mol$ glutaryl-CoA.

Even under physiological conditions where the redox potential of ferredoxin is much lower (–500 mV) and that of NADH could be higher (–280 mV), the Gibbs energy decreases only to $\Delta G' = +10$ kJ/mol, which is still too high for the reaction in this direction. The consecutive decarboxylation of glutaconyl-CoA to crotonyl-CoA ($\Delta G^{\circ\prime}$ = –20 to –30 kJ/mol) could drive the reaction forward, but this energy is expected to generate an electrochemical Na^+ gradient, which brings this reaction to equilibrium ($\Delta G' = 0$).

Initial attempts to investigate glutaryl-CoA oxidation have been carried out using the heterologously expressed genes encoding glutaryl-CoA dehydrogenase and ETF from *S. aciditrophicus* [Djurdjevic, 2010]. Glutaryl-CoA dehydrogenase is encoded by gene SYN_00480 that is located between genes encoding the biotin carboxy carrier, gcdC SYN_00479, and the α-subunit of glutaconyl-CoA decarboxylase, *gcdA* SYN_00481. The recombinant protein (GDH), which shares 73% sequence identity with the glutaryl-CoA dehydrogenase from *Desulfococcus multivorans* [Wischgoll et al., 2009] catalyzed the oxidation of glutaryl-CoA with two $FcPF_6$. In the assay used, the product glutaconyl-CoA was further converted by added glutaconyl-CoA decarboxylase from *A. fermentans* [Buckel, 1986], and the formed crotonyl-CoA was determined with the NADPH-dependent crotonyl-CoA carboxylase/reductase [Erb et al., 2007]. Glutaconyl-CoA inhibited the oxidation of glutaryl-CoA in a noncompetitive manner: $K_i = 34 \pm 10$ μM. This kinetic behavior is observed when the inhibitor binds equally well to the free enzyme and to the enzyme-substrate complex, whereby only V_{max} is decreased and the apparent K_m remains constant. The FAD-containing Gdh_{Syn} catalyzes the oxidation of glutaryl-CoA in two steps with a 'ping-pong' mechanism. In the first step FAD oxidizes glutaryl-CoA to glutaconyl-CoA, and in the second step two Fc^+ reoxidize the $FADH^-$ back to FAD. Hence, the enzyme exists in two states, the oxidized FAD state and the reduced $FADH^-$ state, to which glutaconyl-CoA appears to bind equally well and causes the kinetics of a noncompetitive inhibition.

The ETF from *S. aciditrophicus*, encoded by the genes SYN_02637 (α-subunit) and SYN_02636 (β-subunit), produced in *Escherichia coli* and purified in the presence of 8 μM FAD, catalyzed the reduction of iodonitrosotetrazolium salt to the red formazan by NADH (specific activity 0.4 U/mg) or by NADPH (2.0 U/mg). This activity

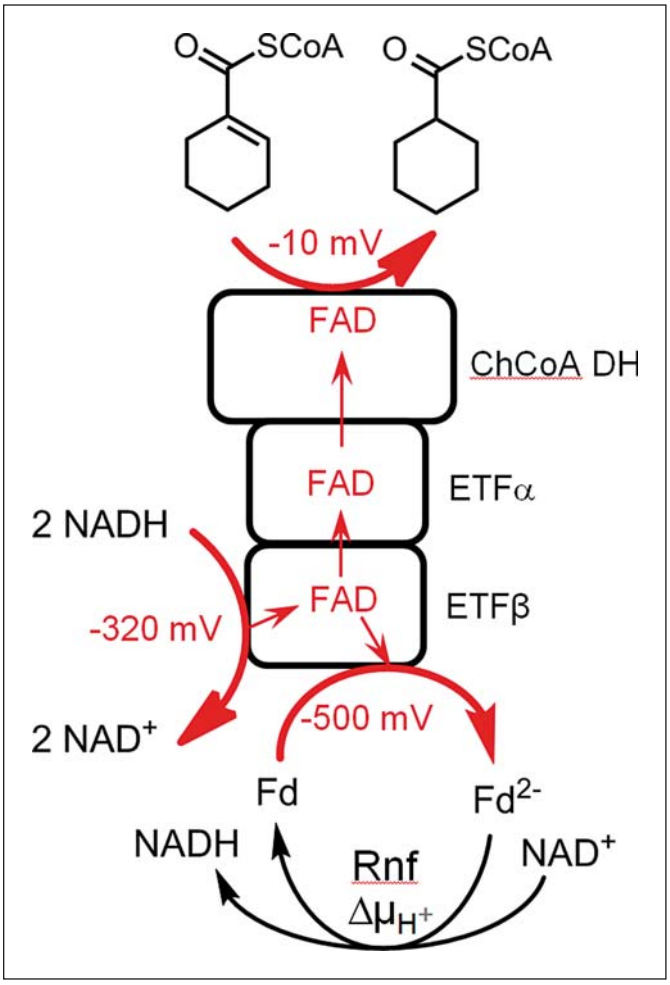

Fig. 5. Hypothetical scheme of reactions involved in electron transfer from NADH to Ch1CoA during cyclohexane carboxylate fermentation in *S. aciditrophicus*. An electron bifurcating ETF transfers electrons from NADH to Ch1CoA (exergonic reaction) and to ferredoxin (endergonic reaction); FAD of the EtfB serves as electron bifurcation site. An Rnf complex catalyzes exergonic electron transfer from reduced ferredoxin to NAD^+ coupled to the transport of an ion (here H^+) across the cytoplasmic membrane.

suggested that this ETF could be involved in electron bifurcation, but in the presence of ETF, GDH, ferredoxin and glutaconyl-CoA no oxidation of NADH could be observed. In agreement with this negative result, ETF does not contain the signature sequence QAIDGDTAQVG near the β-FAD that was found in all known bifurcating ETFs [Chowdhury et al., 2015]. Thermodynamic considerations also lead to the conclusion that bifurcation is not involved in the acyl-CoA dehydrogenases of *S. aciditrophicus*, because the oxidation of the three acyl-CoAs needs to proceed in reverse (see above). Therefore, it ap-

pears more likely that ETF from *S. aciditrophicus* is a nonbifurcating ETF that reversibly transfers electrons from glutaryl-CoA to menaquinone as proposed earlier [McInerney et al., 2007].

Ring-Forming/Cleaving Enzyme

In the benzoyl-CoA degradation pathway the cyclic ring system is cleaved hydrolytically by 2-oxocyclohex-1-ene-1-carboxyl-CoA hydrolase (BamA; fig. 1). It was unclear whether *S. aciditrophicus* employs the identical enzyme in both directions, as ring cleavage is estimated to be exergonic [Kuntze at al., 2008]. The *bamA* gene from *S. aciditrophicus* was heterologously expressed in *E. coli*, and it catalyzed the identical reaction as enzymes involved in the benzoyl-CoA degradation pathway from bacteria with anaerobic respiratory chains [Kuntze et al., 2008]. Though not experimentally demonstrated, it is assumed that BamA from *S. aciditrophicus* catalyzes both: ring cleavage and ring formation. The latter, rather endergonic direction will be driven forward by the rather exergonic reduction of the 6-oxocyclohex-1-ene-1-carboxyl-CoA product to the corresponding alcohol by an alcohol dehydrogenase (BamQ) [Wischgoll et al., 2005].

Crotonyl-CoA Carboxylation

In the benzoyl-CoA degradation pathway of strictly anaerobic bacteria glutaryl-CoA is not oxidatively decarboxylated to crotonyl-CoA and CO_2, but is rather converted to glutaconyl-CoA by a nondecarboxylating glutaryl-CoA dehydrogenase [Wischgoll et al., 2009]. This reaction allows conserving energy by membrane-bound, biotin-dependent glutaconyl-CoA decarboxylase that couples decarboxylation to the transport of ions (H^+/Na^+) across the cytoplasmic membrane [Buckel, 2001a]. The reversibility of Na^+-pumping decarboxylases was demonstrated by inserting methylmalonyl-CoA decarboxylase from *Veillonella parvula* together with oxaloacetate decarboxylase from *Klebsiella aerogenes* into phospholipid vesicles. The reconstituted vesicles catalyzed the Na^+-dependent transcarboxylation of malonyl-CoA to pyruvate, yielding acetyl-CoA and oxaloacetate, as well as the reverse transcarboxylation of oxaloacetate to acetyl-CoA, yielding pyruvate and malonyl-CoA. Both reactions were completely inhibited by monensin, which exchanges Na^+ by H^+ [Hilpert and Dimroth, 1984]. Hence, it is reasonable to propose that the glutaconyl-CoA decarboxylase from *S. aciditrophicus* works in both directions. Four genes, *gcdABCD*, encode the glutaconyl-CoA decarboxylases from several anaerobes [Kress et al., 2009]. In *S. aciditrophicus* only *gcdABC* (*SYN_00481, SYN_01431, SYN_00479*) are present, whereas *gcdD* is missing. GcdA, the carboxytransferase, is a 65-kDa protein that catalyzes the transfer of the carboxyl group of glutaconyl-CoA to biotin attached to GcdC, the biotin carrier protein. Instead of GcdC, free biotin can also work as an acceptor, though at a much lower rate. The very hydrophobic GcdB acts as the actual Na^+ pump which is driven by the decarboxylation of the carboxybiotin of GcdC. GcdD is thought to act as a stabilizer of the GcdABCD complex [Buckel, 2001a]. Recombinant pure GcdA from *S. aciditrophicus* indeed mediated the carboxyl transfer with free biotin [Kim, 2011]. The specific activity, measured by the formation of crotonyl-CoA from glutaconyl-CoA (see above), was very low (2 mU/mg protein in the presence of biotin and 0.6 mU/mg in its absence). For comparison, with GdcA from *A. fermentans* a specific activity of 40 mU/mg was achieved: 1% of that of the holoenzyme [Buckel and Liedtke, 1986]. The GcdB and GcdC components could not be produced in *E. coli*.

Cyclohexane Carboxylate Fermentation versus Degradation

The catabolic crotonate and/or benzoate degradation pathways in *S. aciditrophicus* can be referred to as cyclohexane carboxylate fermentation – in analogy to other fermentations that are named according to their released end products. During axenic growth with crotonate, it makes use of the benzoyl-CoA degradation pathway enzymes together with at least three additional steps catalyzed by two acyl-CoA dehydrogenases and a so far unknown enzyme involved in the conversion of ChCoA to cyclohexane carboxylate.

Genes with high similarities to ChCoA and Ch1CoA dehydrogenases (67% and ≥71%, respectively) are present in genomes of other Deltaproteobacteria. However, none of these organisms is known for fermentative growth. They comprise metal oxide- or sulfate-respiring organisms belonging to the genera *Geobacter*, *Desulfococcus* or *Desulfobulbus*. In these species, the encoding genes of the two acyl-CoA dehydrogenases are arranged in gene clusters that additionally contain genes encoding putative CoA transferases (*Geobacter* species) or putative carboxylic acid CoA ligases (sulfate-reducing species); they are under the control of transcription regulators (fig. 6). In respiring organisms these genes are used for complete cyclohexane carboxylate degradation, as demonstrated for *G. metallireducens*. In this organism a succinyl-CoA:cyclohexane carboxylate CoA transferase activates

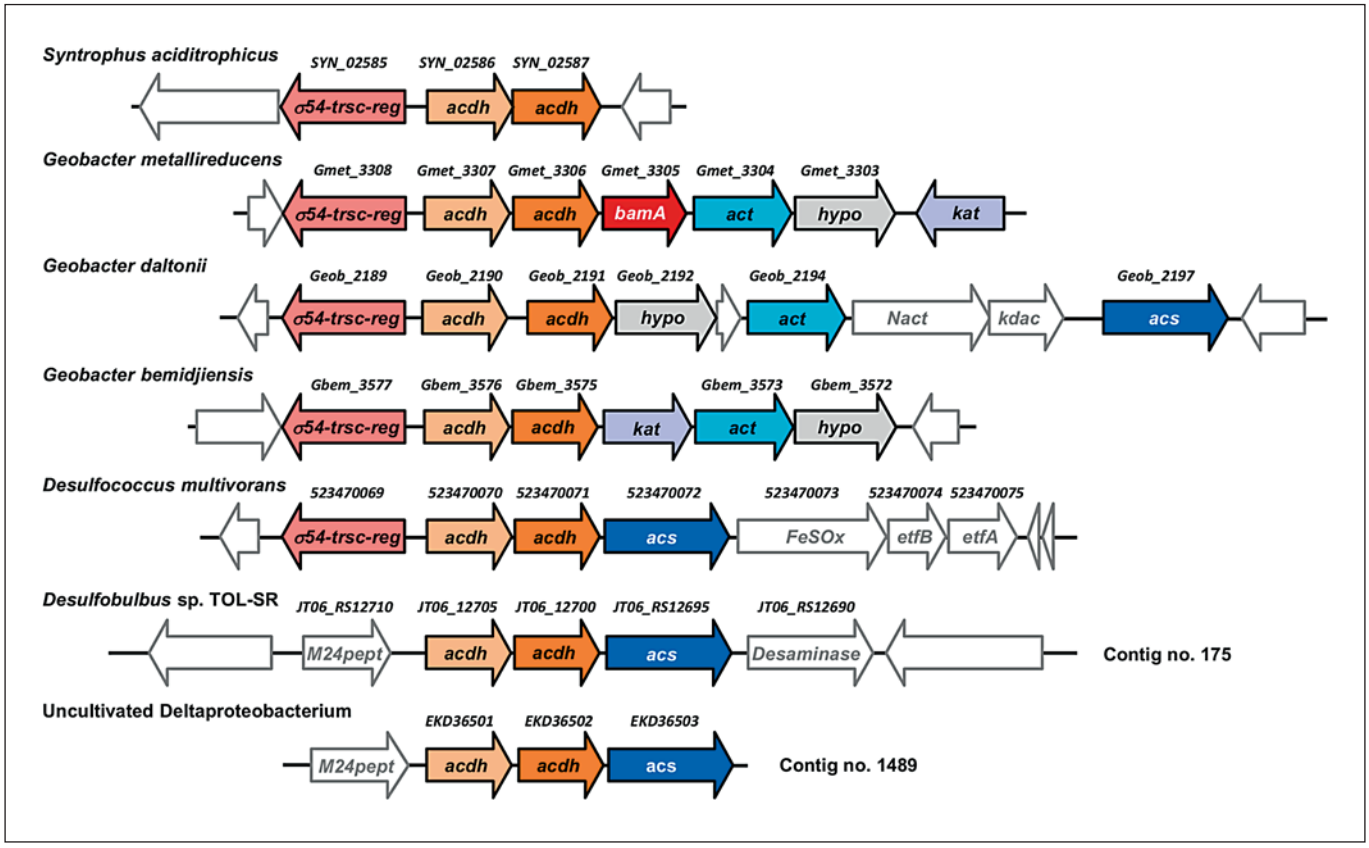

Fig. 6. Gene clusters involved in cyclohexane carboxylate metabolism in Deltaproteobacteria. σ54-trsc-reg = Sigma54-dependent transcription regulator; acdh = acyl-CoA dehydrogenase; act = acyl-CoA transferase; hyp = hypothetical protein; kat = ketoacyl-CoA thiolase; Nact = N-acetyltransferase; kdac = zinc-dependent lysine deacetylase; acs = acyl-CoA synthetase; FeSOx = FeS oxidoreductase; etf = electron transferring flavoprotein; M24pept = Met24 peptidase. Genes with high sequence similarities are displayed in the same color: homologs of the ChCoA and Ch1CoA dehydrogenases (light and dark orange), proteins annotated as rσ54-dependent regulators (pink), homologous CoA transferases (light blue), homologous CoA ligases (dark blue), highly similar hypothetical proteins (grey). The gene cluster in *G. metallireducens* contains an additional *bamA* gene encoding a ring-opening hydrolase (red).

the substrate [Kung et al., 2014]. In *S. aciditrophicus* the ChCoA and Ch1CoA dehydrogenases are expected to play a crucial role in both axenic cyclohexane carboxylate formation and syntrophic cyclohexane carboxylate oxidation [Kung et al., 2013]. However, whether identical enzymes are involved in cyclohexane carboxylate activation and release from ChCoA is unknown.

The cyclohexane carboxylate degradation pathway in obligate anaerobes proceeds via the Ch1,5CoA key intermediate and differs from the one reported for the phototrophic *Rhodopseudomonas palustris* [Perrotta and Harwood, 1994]. Here, the Ch1CoA intermediate is hydrated to 2-hydroxycyclohexane-1-carboxyl-CoA rather than being oxidized to Ch1,5CoA. Modified reactions of the benzoyl-CoA degradation pathway lead to aliphatic pimeloyl-CoA instead of 3-hydroxypimeloyl-CoA. The ChCoA dehydrogenase of *R. palustris* involved in this pathway shows only 29% amino acid sequence identity with the ChCoA dehydrogenase from *S. aciditrophicus*. This finding suggests that this pathway evolved independently of the cyclohexane carboxylate degradation pathway of Deltaproteobacteria [Kung et al., 2014]. It is evident that all bacteria that degrade cyclohexane carboxylate can also grow with aromatic compounds but not vice versa [Aklujkar et al., 2010; Peters et al., 2004; Prakash et al., 2010; Wischgoll et al., 2005]. In conclusion, the capability to degrade/form cyclohexane carboxylate most probably evolved as an add-on to an already existing benzoyl-CoA degradation pathway at the Ch1,5CoA level [Kung et al., 2014].

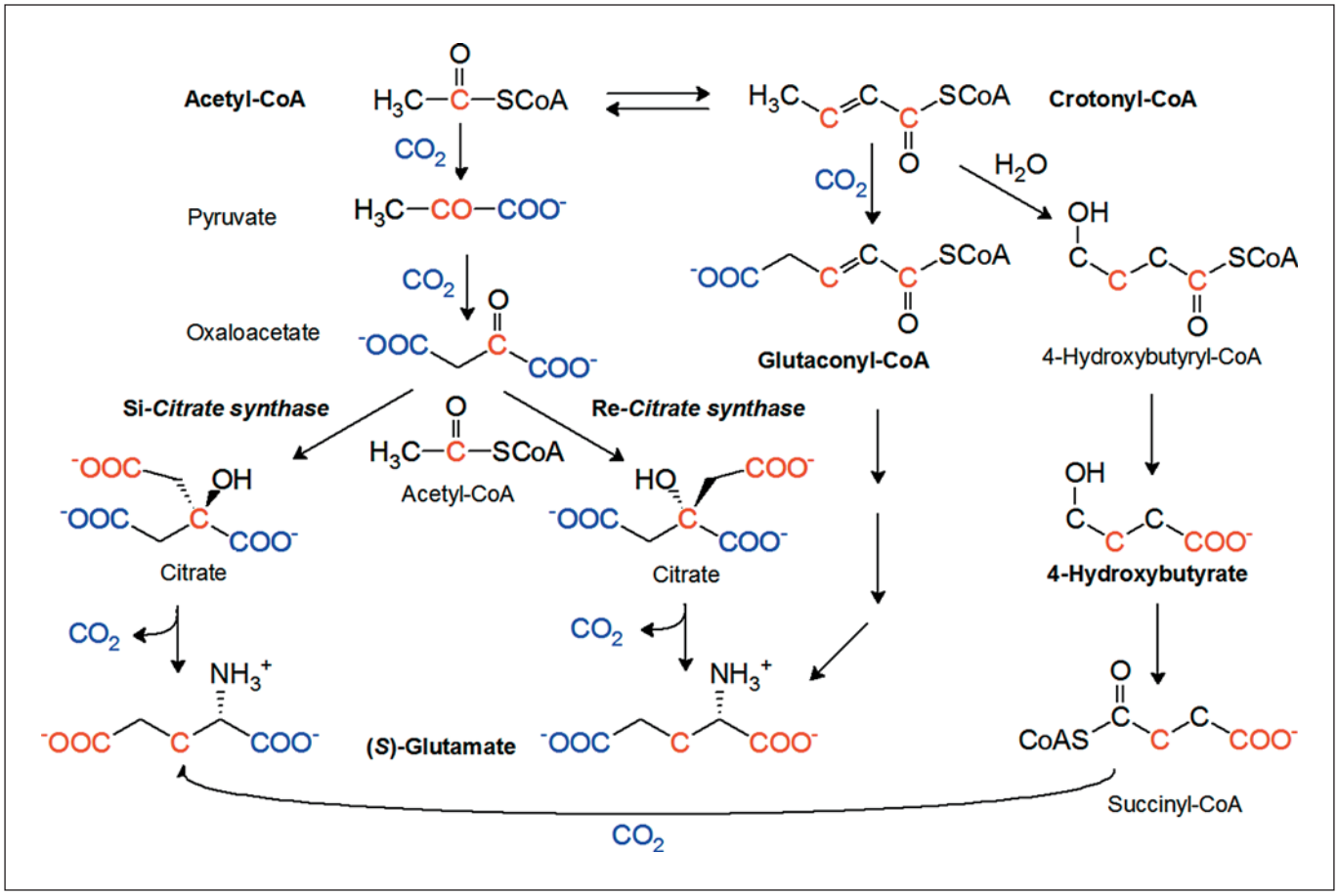

Fig. 7. Possible pathways in *S. aciditrophicus* leading from labeled acetyl-CoA (crotonyl-CoA) and CO_2 to labeled glutamate; for details see text.

Synthesis of Glutamate in *S. aciditrophicus*

The classical pathway of the biosynthesis of *(S)*-glutamate proceeds via citrate, isocitrate and 2-oxoglutarate. In the genome of *S. aciditrophicus*, however, no *gltA* gene encoding *Si*-citrate synthase was found [McInerney et al., 2007]. The other genes for this pathway, starting from acetyl-CoA by two carboxylations to pyruvate (pyruvate-ferredoxin oxidoreductase) and oxaloacetate (pyruvate carboxylase), as well as the genes of the oxidative branch of the Krebs cycle comprising aconitase, isocitrate dehydrogenase (decarboxylating) and glutamate dehydrogenase, are present in the genome [McInerney et al., 2007]. Therefore, alternative routes were examined that could use *Re*-citrate synthase, 2-hydroxyglutaryl-CoA dehydratase (glutaconyl-CoA hydratase) or 4-hydroxybutyryl-CoA dehydratase (crotonyl-CoA hydratase) [Buckel, 2001b]. The *Re*-citrate synthase from *C. kluyveri* [Li et al., 2007] shares 49% amino acid sequence identity to a putative homocitrate/isopropylmalate/citramalate synthase in *S. aciditrophicus* (gene SYN_02536). The gene was expressed in *E. coli* and the product was identified as a highly specific homotetrameric *Re*-citrate synthase, which contained one Ca^{2+} per 72-kDa subunit [Kim et al., 2013]. The activity of the oxygen-insensitive enzyme of 0.7 U/mg was stimulated twofold by Mn^{2+} and threefold by Co^{2+}, but completely abolished by EDTA. To assess the action of *Re*-citrate synthase in the glutamate synthesis of *S. aciditrophicus* during axenic growth with crotonate, labeling studies with [1-^{14}C]acetate, [1-^{13}C]acetate and [^{13}C]bicarbonate were performed [Kim et al., 2015]. The ^{13}C-labels detected in all eight amino acids identified by gas chromatography-mass spectrometry were consistent with the idea that in *S. aciditrophicus* all biosyntheses start with acetate and CO_2. The experiments with [1-^{14}C]acetate and [1-^{13}C]acetate revealed the incorporation of label into the C1 of gluta-

mate, as expected for a pathway via *Re*-citrate synthase or glutaconyl-CoA (fig. 7). The latter route could be excluded as the hydration of glutaconyl-CoA to (*R*)-2-hydroxyglutaryl-CoA is an endergonic reaction with $\Delta G^{\circ\prime} = +19.9$ kJ/mol [Parthasarathy et al., 2010], and as only two of the three genes coding for the heterodimeric dehydratase and the homodimeric activator are present in the genome. Moreover, genes for CoA transferase/thioesterase and (*R*)-2-hydroxyglutarate dehydrogenase are lacking. However, the quantitative incorporation of [1-^{13}C]acetate as well as ^{13}C-NMR measurements on glutamate isolated from $NaH^{13}CO_3$-labeled cells also revealed the incorporation of ^{13}C into the C1 of glutamate, which indicates participation of a pathway via succinate that could be derived from 4-hydroxybutyryl-CoA dehydratase (fig. 7). Although a silylated derivative of 4-hydroxybutyrate has been detected by gas chromatography-mass spectrometry in *S. aciditrophicus* cells, the putative 4-hydroxybutyryl-CoA dehydratase (gene SYN_02445) cannot catalyze the hydration of crotonyl-CoA due to the lack of two glutamates and one tyrosine in the active site, whereas the residues coordinating the [4Fe-4S] cluster (3 cysteines and one histidine) and interacting with the N5 of the FAD cofactor (threonine) are present [Zhang et al., 2015]. Modeling studies with the crystal structure of the well-characterized *Clostridium aminobutyricum* enzyme [Martins et al., 2004] also indicate that the *S. aciditrophicus* protein must have another function. Perhaps the elucidation of the function of this protein may elucidate the unresolved glutamate synthesis pathway in *S. aciditrophicus*.

Outlook

S. aciditrophicus has been established as the best-studied model organism for the anaerobic metabolism of aromatic compounds in a fermenting organism. So far this organism is unique in degrading carboxylic acid carbon sources to cyclohexane carboxylate as a fermentation product. Future work will address the unresolved question of how electrons from NADH are transferred to enoyl-CoA for NAD^+ regeneration. ETF and ferredoxin could play a role during axenic cyclohexane carboxylate fermentation, but also in the reverse reaction, where cyclohexane carboxylate is oxidized to acetate during syntrophic growth. The latter scenario is related to one of the most intriguing questions in syntrophy: how are the high-potential electrons derived from acyl-CoA oxidation to enoyl-CoA ($E^{\circ\prime} \approx -10$ mV) transferred to reduce protons to H_2 or CO_2 to formate against a huge thermodynamic barrier of $\Delta E' \approx -250$ mV (considering syntrophic concentrations of the formate/H_2 products)? The often suggested involvement of menaquinones and the membrane potential has not been experimentally demonstrated to date [Worm et al., 2014]. A gene annotated as an FeS oxidoreductase, *oxr*, is located next to the two encoding genes of ETF, *etfAB*. It is likely that it plays a crucial role in transferring electrons from acyl-CoA to CO_2/H^+.

The presence of five BamBCDEF components of class II BCRs asks the question of their function. They could serve as back-up genes or, as an alternative, they could function as isoenzymes with, for example, altered substrate specificity. The latter scenario is known from class I BCRs. Though the structure of the active site BamBC of a class II BCR has recently been solved [Weinert et al., 2015], the question of which process drives the endergonic electron transfer from reduced ferredoxin to the aromatic ring is still unresolved.

Acknowledgments

W.B. thanks Dr. Ivan Berg (University of Freiburg) for suggesting the pathway via 4-hydroxybutyrate for the synthesis of glutamate in *S. aciditrophicus*. This work was funded by the Deutsche Forschungsgemeinschaft (DFG) within the framework of the Scientific Priority Programme SPP 1319 'Biological transformations of hydrocarbons without oxygen: from the molecular to the global scale'.

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